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13. ABSTRACT *(Maximum 200 words)*

Rational approaches to successful prostate cancer immunotherapy requires fundamental knowledge of mechanisms whereby tumor antigens are acquired, processed, and immune responses generated. Extensive heterogeneity of MHC Class I and II molecules affords the human population broad immune protection against infectious diseases, however, creates restrictions for cancer immunotherapy. These restrictions center around antigenic determinants generated from potential tumor-associated antigens. This project seeks to determine candidate prostate tumor antigens as an approach to developing rational immunotherapy. One candidate protein for study is prostate specific antigen (PSA). We found CD4+ T-cell responses to PSA in some men with chronic prostatitis, a presumed autoimmune disorder. Methods for isolating antigen presenting cells (DCs) were developed that: a) acquired antigen from apoptotic prostate cancer cell lines and induced T-cell responses in vitro; b) acquired PSA and PSA/anti-PSA complexes and generated CD8+ and CD4+ T-cell responses in vitro. These results suggest that PSA/anti-PSA complexes may be an immunotherapeutic approach for prostate cancer. To be effective, "prostate cancer antigen" specific cytotoxic T-cells must recognize antigen presented in the context of HLA molecules on the tumor cells. We show that prostate cancer cell lines (except LNCaP) express antigen processing and transport genes that were upregulated by interferon-γ.

14. SUBJECT TERMS
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Dean Allen, MD 7/22/99

PI - Signature Date

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5. Introduction

HLA Class I and Class II molecules present peptides derived from proteins from exogenous and endogenous sources in the generation of an immune response. In tumor immunity, the most likely sources of these proteins are from products that are overproduced by the malignant cell and/or when the tumor cell undergoes apoptosis. The immune system recognizes products of the malignant cells that have amino acid changes that differ from normal proteins. In general, a tumor cell does not serve as primary antigen presenting cell to initiate an immune response. Rather the immune response must be initiated by a primary antigen presenting cell, called the dendritic cell (DC). Potential tumor antigens can be acquired by DC's by several mechanisms, most likely being the uptake of tumor cell fragments when these cells undergo apoptosis. Secreted cellular products are not generally acquired by DC unless they are altered in such a fashion that allows interaction with receptors on the DC. Once an immune response is established to a specific cellular product, the antigen must be exhibited by the Class I molecule on the surface of a malignant cell in order for that cell to be targeted for immune elimination.

There is an increasing body of data that indicates that tumors may escape immune recognition due to lack of expression of Class I (or Class II) molecules on the tumor cell surface. There are a number of mechanisms that may account for this failure including decreased to absent expression of genes that are involved in the intracellular antigen processing which is required for these molecules to be expressed on the cell surface.

Our objectives during the first year of this grant were to establish laboratory methods that would: (1) allow consistent and reproducible isolation of DC precursors from peripheral blood lymphocytes; (2) determine methods to arm dendritic cells with products of prostate tumor cells or with prostrate specific antigens; and (3) establish methods that would measure T-cell responses engendered by antigen armed DC's. In addition, we have investigated the expression of antigen processing (LMP-2, LMP-7) and transport genes (TAP-1, TAP-2) in prostate cancer cell lines and cell lines established from normal prostate tissue.

6. Body

Dendritic Cell Isolation and Characterization

Functional primary antigen presenting cells (DC) may be derived from myeloid (pDC1) and lymphoid (pDC2) precursors in peripheral blood. pDC1's express CD14, CD11c, and HLA-DR, are the most accessible, and promote the TH1 responses that are desired in our studies. As illustrated in Figure 1, cells with these marker combinations were found predominantly in the region designated as R1 in a side scatter flow cytometry plot of mononuclear cells isolated from peripheral blood on density gradients. These mononuclear cells were obtained from peripheral blood apheresis product from a normal volunteer with the HLA-A*0201 allele and cryopreserved in RPMI-1640 containing 40% human serum and 10% DMSO. We used two approaches in attempts to isolate pDC1 dendritic cell precursors. The first approach was to sort these cells by flow cytometry, selecting those cells who were positive for the triple markers or alternatively that were discriminated by size in the forward and side scatter plot. Relatively pure amounts of the DC precursors (based on the expression of the above triple marker combination) were obtained using either of the above methods. The yields were ranged from 30-50% of expected, however, these cells matured poorly under the influence of cytokines and did not maintain viability in culture. We have developed a third technique which is highly reproducible and yields sufficient numbers of dendritic cell precursors in order for us to pursue our studies.

Peripheral Blood Mononuclear Cells (PBMCs) that have been viably frozen were thawed and plated at 10^7 cells/well into 6 well plates. The cells were allowed to adhere by incubation at 37°C for 2 hr or overnight. Following the incubation, the plates were gently swirled and the non-adherent cells were removed. The wells were gently washed once with RPMI containing 10% human AB serum. The adherent/loosely adherent cells were cultured for 4 days in 2 ml of the above media supplemented with 1000 Units/ml GM-CSF and 1000 Units/ml IL-4. On day 4, the cells were pulsed with antigens (described below) or exposed to prostate tumor cell lines that had been induced to undergo apoptosis, incubated for 8 hr and 100 μg/ml of CD40 ligand was added to the cultures. After 3 days of culture, the cells were harvested by vigorously washing the wells twice with RPMI and once with PBS. The dendritic cells were analyzed by flow cytometry for surface cell markers and cultured with autologous PBMC for functional assays.

Figure 2 shows an example of the surface antigens expressed on the loosely adherent population of cells that were cultured with GM-CSF and IL-4 cytokines and matured with CD40 ligand. As can be seen in this figure, approximately 70% of the total cell population had the forward and side scatter characteristics as well as some of the surface antigen markers of dendritic cells.

Preparation of Apopotic Prostate Tumor Cell Lines and PBMC Response

Three cell lines were used in these studies: LNCaP, DU-145, and PC-3. We attempted to induce apoptosis in these three cell lines by gamma irradiation, deprivation of sera in culture media for 24-48 hr alone followed by gamma irradiation and with UVB irradiation. The readout for apoptosis was the appearance of annexin V as measured by flow cytometry. The most effective means of inducing apoptosis was with the combination of sera deprivation and gamma irradiation. However, in these circumstances only 10-20% of the cells appear to be apoptotic. Supernatants from these cells were used as antigen preparations for arming of dendritic cells.

Table 1 shows an example of results obtained from arming dendritic cells with apoptotic prostate tumor cell lines. As can be seen from this table, this method appears to effectively stimulate lymphocyte responses, however, there is a definite lack of specificity in the recall response.

T-cell Activation by Prostate Specific Antigen

The other source of antigen(s) that were used to investigate antigen presentation by DC in these studies are prostate specific antigen (PSA) with and without a monoclonal antibody to this protein (αPSA) (kindly provided by the AltaRex Corp). PSA and αPSA were added at a concentration of 10 μg/ml to the DC prior to maturation with CD40 as were equimolar concentrations of the PSA and αPSA. After 72 hr, 5×10^4 DC were added to 1×10^6 freshly thawed PBMCs in 24 well plates in 1 ml culture media. The plates were cultured for 4 or 7 days, harvested, and restimulated with antigen and cultured overnight in 12 x 75 snap-top tubes. T-cell activation was measured by the expression of CD69 on CD4 and CD8+ T-cells using flow cytometry. Proliferation assays were performed by plating 2.5×10^4 cells/well into 96 well U-bottom plates and cultured for 5 days with ^{3}H-thymidine added for the final 8 hr of culture. Cells were harvested on glass fiber filter mats and ^{3}H-thymidine incorporation determined by solid phase scintillation counting.

Table 2 shows the results of T-cell proliferation and CD4 and CD8 T-cell activation by

DC presenting PSA, αPSA, and the PSA/αPSA complex. T-cell proliferation and CD4+ and CD8+ T-cell activation were measured at 4 days and 7 days following cultures of PBMCs alone, with unarmed DC and DCs armed with the above antigens. Proliferative responses and activation of both CD4+ and CD8+ T-cells were observed in the PBMCs cultured for 4 days then stimulated with PSA and the PSA/αPSA combination, but not when these PBMC were cultured for 7 days. The same pattern of responses were found when the unarmed DC were added to 4 day cultures where only the PSA/αPSA combination activated both CD4 and CD8+ T-cells. The increase in baseline proliferation and activation that occurred when the DCs were added to cultures is most probably explained by the well-known phenomena of the autologous mixed lymphocyte reaction. Proliferation and CD4+ and CD8+ T-cell activation was found with PBMC cultured with DC armed with PSA or with PSA/αPSA. Restimulation with PSA and with PSA/αPSA increased both the levels of activation and numbers of CD4+ and CD8+ cells. At 7 days, PBMC proliferated in response to exposure only to the PSA/αPSA combination. Addition of DC's armed with PSA increase the baseline responses with further activation by restimulation with PSA or with the PSA/αPSA complex in the 4 day cultures. However, only the antigen antibody combination restimulated the PBMC's at 7 days. These results demonstrate the efficacy of the in vitro T-cell activation by dendritic cells presenting antigenic determinants in PSA. We have compared the ability of DC's to present PSA alone or when the PSA and anti-PSA were added together. This approach was taken to determine if T-cell activation was augmented by introducing antigen in the form of an antigen antibody complex. Based on the results found in these experiments, both T-cell proliferation and activation was observed when the antigen activated cells were restimulated with the antigen antibody complex. There appears to be no preferential activation of CD4+ or CD8+ T-cells indicating that the antigens are being processed through both the Class I and Class II pathways. The next steps in our studies will be to determine the specific peptides presented by the HLA Class I and Class II molecules in the prostate specific antigen.

Autoimmunity shares many of the elements that might be desired in the immunotherapy of prostate cancer. Determination of antigens that are targets of autoimmune diseases of the prostate may afford insight into the potential antigens that might be used to generate immune responses to prostate cancer. The ability of peripheral blood mononuclear cells to respond to seminal plasma proteins was examined in 14 patients with chronic prostatitis, chronic pelvic pain, and 12 normal volunteers. Greater than two-fold increase in proliferative response to PSA was observed in 36% of patients and no significant response in the normal volunteers. The details of this study are reported in an appended manuscript submitted for consideration for publication to the Journal of Urology.

We investigated the expression of antigen processing and transport genes in cell lines established from freshly explanted normal prostate epithelium and primary prostate carcinoma as well as several well known long-term cultured prostatic cancer cells. Low level expression of these genes was found in the normal and prostate cancer cells. In the long-term cell lines, these genes were expressed at very low to undetectable levels. Addition of IFN-γ upregulated the expression of the genes in each of the cell lines except in LNCaP. The details of these studies are reported in an appended manuscript draft.

7. Key Research Accomplishments

- Isolation, maturation of primary antigen presenting cells (dendritic cells, DC) from precursors in peripheral blood mononuclear cells.

- DCs present antigens from apoptotic prostate cancer cell lines.
- CD4+ and CD8+ T-cell responses were induced by mature DCs armed with PSA and PSA/anti-PSA complex.
- CD4+ lymphocytes from some patients with chronic prostatitis/chronic pelvic pain syndrome respond to PSAs suggesting an autoimmune etiology.
- Paired normal prostate epithelium and primary cancer cells from the same patients were shown to express antigen processing in peptide transport genes that could be upregulated by interferon-γ. These genes were also expressed in 2 of 3 long-term prostate cancer cell lines.

8. Reportable Outcomes

Two (2) manuscripts prepared for submission are appended.

9. Conclusions

Immunotherapy of prostate cancer may serve as an effective adjunct to current treatment modalities. In order to develop this treatment, certain fundamental questions need to be addressed. Autoimmune disease has many features that are similar to objectives that may be sought for successful immunotherapy of cancer. Observation that immune response to PSA can be documented in some men with chronic prostatitis suggest non-immune etiology of this disease.

Efficient reproducible methods that deliver prostate tumor antigens to induce immunity are needed. Primary antigen presenting cells (DC) armed in vitro with tumor antigens or peptides have been used to generate anti-tumor immunity in animal models and more recently in man. We have successfully procured DC precursors from peripheral blood lymphocytes from normal individuals matured and armed these cells to present antigen acquired from apoptotic prostate cancer cell lines, PSA, and PSA/anti-PSA antigen antibody complexes. The rationale for the use of the latter was to take advantage of high affinity Fc receptors on DCs. We hypothesized that antigen uptake mediated through these receptors would increase the efficiency of antigen uptake. **The demonstration that CD4+ T-helper responses and CD8+ T-cell (cytotoxic cell precursors/effectors) can be generated by PSA/anti-PSA complexes offers a rationale for a potential therapeutic trial.**

Once cytotoxic T-cells are generated, tumor cells must present the immunogenic peptide in a Class I molecule on the surface in order for killing to occur. Lack of Class I expression has been documented in a number of different tumor types. One mechanism which accounts for this circumstance is the absence of expression of the antigen processing and intracellular transport genes that are obligatory for HLA Class I expression at the cell surface. We demonstrated that these genes are expressed in cell lines recently established from normal prostate epithelium and prostate cancer from the same patient. These studies suggest that prostate cancer cells may be suitable targets for cytotoxic T-cells that are generated in immunotherapy trials.

10. References

None

11. Appendices

Figures 1 and 2; Tables 1 and 2; Two preprints

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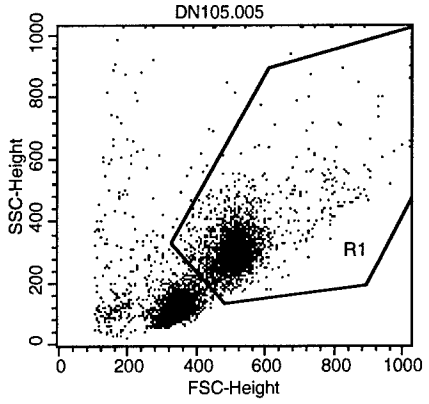
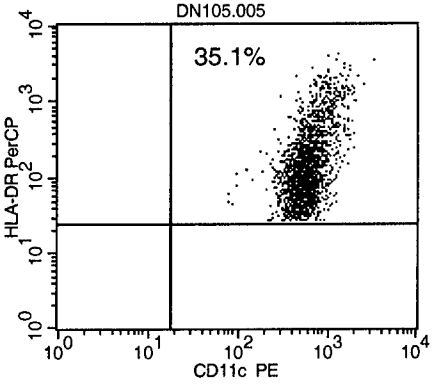
Figure 1

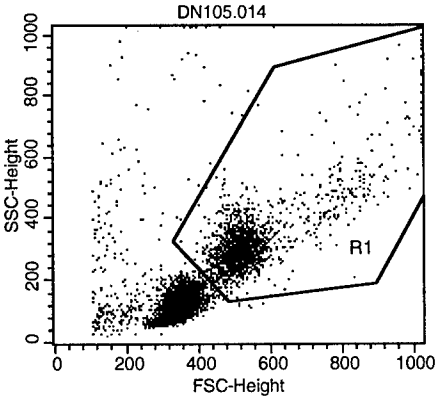
Normal HLA - A2
Donor



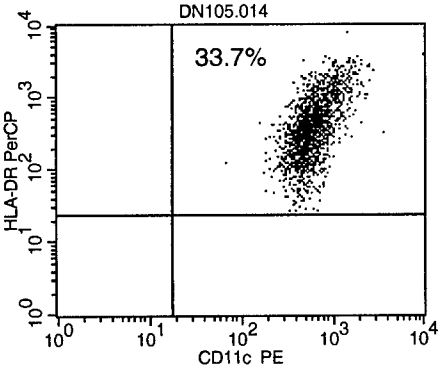
R1 = 48.4% of Total



14/11c/DR + in R1
of PBMCs

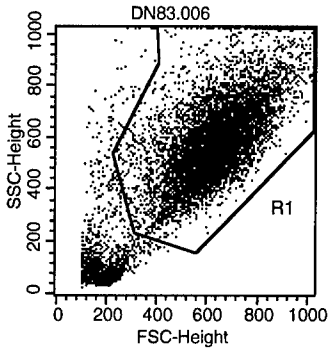


R1 = 37.5% of Total



14/11c/DR+ in R1
of Nonadherent PBMCs
after 2hr plastic
adherence

HLA-A2 Donor DC preparation



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Region	Events	% Total
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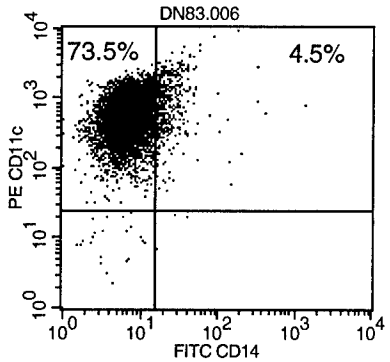

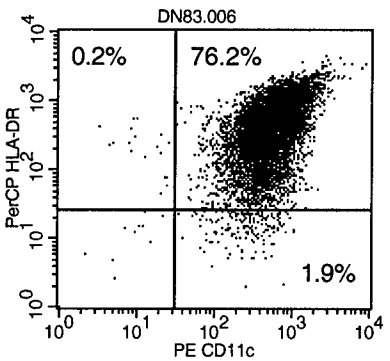

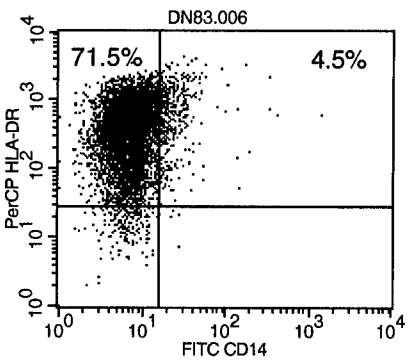

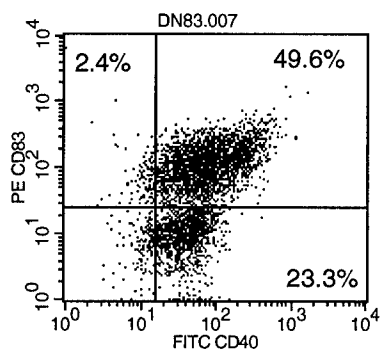

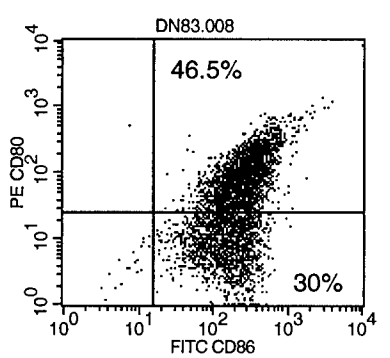

Table1. Proliferation of PBMC in Response to DC Presentation of Apoptotic Prostate Cancer Tumor Cell Lines.[1]

Stimulator	Responder	Restimulation	CPM
DC/Du-145	PBMC	O	1160
"	"	Du-145	9829
"	"	LNCaP	439
"	"	PC-3	2850
DC/LNCaP	"	O	304
"	"	Du-145	29086
"	"	LNCaP	93
"	"	PC-3	2365
DC/PC-3	"	O	247
"	"	Du-145	18755
"	"	LNCaP	107
"	"	PC3	4645

1) Induction of apoptosis described in the Methods Section.

Table 2. T-cell Proliferation and CD4+ and CD8+ T-cell Activation by Dendritic Cells Presenting PSA, Anti-PSA maAb, and PSA/αPSA.

			4 Days			7 Days		
Stimulator	Responder	Restimulation	CPM	% CD69+ CD4+	% CD69+ CD8+	CPM	% CD69+ CD4+	% CD69+ CD8+
O	PBMC	O	1213	5	11	240	4	5
O	PBMC	PSA	2304	12	16	268	2	6
O	PBMC	αPSA	770	6	13	141	2	5
O	PBMC	PSA/αPSA	2352	12	14	233	6	5
DC	PBMC	O	2749	20	30	1934	8	9
DC	PBMC	PSA	27680	39	42	1587	6	10
DC	PBMC	αPSA	2575	21	29	1690	7	9
DC	PBMC	PSA/αPSA	22249	41	42	2258	19	21
DC-αPSA	PBMC	O	3590	7	13	509	8	11
DC-αPSA	PBMC	PSA	14824	17	24	487	8	4
DC-αPSA	PBMC	αPSA	3068	9	16	481	9	12
DC-αPSA	PBMC	PSA/αPSA	13029	17	25	571	9	13
DC-PSA	PBMC	O	10235	18	25	5791	43	24
DC-PSA	PBMC	PSA	24267	20	26	3359	42	24
DC-PSA	PBMC	αPSA	5371	15	22	5723	32	22
DC-PSA	PBMC	PSA/αPSA	20100	26	31	11195	44	28
DC-PSA/ αPSA	PBMC	O	2533	15	28	5879	36	24
DC-PSA/ αPSA	PBMC	PSA	21544	26	31	5456	39	26
DC-PSA/ αPSA	PBMC	αPSA	3741	15	27	6193	22	23
DC-PSA/ αPSA	PBMC	PSA/αPSA	24337	25	27	16695	45	29

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PSA is a candidate self antigen in auto-immune chronic prostatitis/chronic pelvic pain syndrome

Sathibalan Ponniah, Ifeyinwa Arah and Richard B. Alexander

Division of Urology, University of Maryland School of Medicine and Section of Urology, VA Maryland Health Care System, Baltimore, Maryland

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Running Title: Antigens in autoimmune prostatitis

Keywords: Prostatitis, Prostate specific antigen, autoimmunity, T lymphocytes

Abstract

Purpose: Previous studies have demonstrated that recognition of seminal plasma antigens can occur in patients with chronic prostatitis/chronic pelvic pain syndrome (Alexander et al, Urology 50:893-899, 1997). This suggests that an autoimmune component may contribute to symptoms in some men. To determine if any of the principal secretory proteins of the prostate could be candidate antigens in autoimmune prostatitis we examined the recall proliferative response of purified CD4 T cells in patients with chronic prostatitis/chronic pelvic pain syndrome and normal volunteers using purified seminal plasma antigens and autologous dendritic cells.

Methods: Peripheral blood mononuclear cells were harvested from 14 patients with chronic prostatitis/chronic pelvic pain syndrome and 12 normal volunteers by density gradient centrifugation from peripheral blood samples. The stimulating cells were irradiated autologous dendritic cells produced by culture of monocyte enriched fractions with IL-4 and GM-CSF. Purified CD4 T cells were the responding population. Recall proliferation assays were performed using purified seminal plasma proteins as antigens.

Results: In 14 patients with chronic prostatitis/chronic pelvic pain syndrome we detected a greater than 2 fold increase in proliferative response to PSA compared to control in 5 patients (36%). No response to PAP or β-microseminoprotein was observed in these 14 patients. In 12 normal volunteer donors with no history of genitourinary disease or symptoms no proliferative response above background was observed to any prostatic antigen.

Conclusions: The data suggest that some men with the symptoms of chronic prostatitis/chronic pelvic pain syndrome have evidence of a proliferative CD4 T cell response to PSA. PSA is a candidate antigen in chronic prostatitis/chronic pelvic pain syndrome and may be an appropriate target for immunotherapy for prostatic cancer.

Introduction

Chronic prostatitis/chronic pelvic pain syndrome is a common diagnosis but very little is understood about the etiology of the disease. Men with this syndrome present with an episodic and relapsing condition characterized principally by pain in the pelvic region, voiding symptoms and effects on sexual function (1). These symptoms cannot be distinguished from those of men with acute bacterial infections of the prostate gland, however, the overwhelming majority of men with chronic symptoms cannot be demonstrated to have bacterial infection (2). The disease represents a major problem in the U.S. resulting in 2 million office visits yearly to primary care physicians and urologists annually (3). Most of these men are treated with prolonged courses of antimicrobials with unknown and doubtful benefit.

We recently reported that some men with chronic prostatitis/chronic pelvic pain syndrome have evidence of CD4 T lymphocyte recognition of seminal plasma derived both from normal men and men with seminal vesicle atresia (4). We used seminal plasma as the source of antigens in that study because a significant proportion of the volume of the semen is contributed by the prostate. In addition, semen from men with seminal vesicle atresia consists almost entirely of the secretions of the prostate and is characterized by azoospermia, absent fructose and low volume. These data suggested that some men with chronic prostatitis/chronic pelvic pain syndrome could have an autoimmune component to their disease that could be either a cause or consequence of their symptoms.

To further define the potential antigens contained within the seminal plasma that could be recognized in these patients we studied patients and normals using purified seminal plasma proteins of prostatic origin. The major secretory proteins of the prostate contained within the seminal plasma are prostate specific antigen (PSA), prostatic acid phosphatase (PAP) and β-

microseminoprotein (β-MSP) (5). These proteins, purified from human seminal plasma were used as antigens in CD4 T cell recall proliferation assays in patients with chronic prostatitis/chronic pelvic pain syndrome and normal volunteers. We found that some men with chronic prostatitis/chronic pelvic pain syndrome had a CD4 T cell proliferative response to PSA and that this was not observed in normal volunteers. This suggests that PSA is a candidate antigen in autoimmune chronic prostatitis/chronic pelvic pain syndrome.

Methods

Culture Medium

Culture medium consisted of RPMI 1640 (GIBCO-BRL,) supplemented with 10% human AB serum (heat inactivated) (Gemini Bioproducts, Calabasas, CA), 2% L-glutamine and Penicillin/Streptomycin (Biofluids, Rockville, MD).

Preparation of cell populations

Fifty ml of peripheral blood was drawn from each normal volunteer and prostatitis patient into a syringe containing 1000 units of heparin. The blood samples were centrifuged over a density gradient of Lymphocyte Separation Medium (ICN Biomedicals/Cappel, Aurora, OH) to obtain the peripheral blood mononuclear cell (PBMC) population. The PBMC were then washed, resuspended at $5x10^6$ cells per ml. in a 1:2 dilution of culture medium and PBS. The cells were then centrifuged over a 40% Percoll (Pharmacia, NY) gradient made up in PBS containing 5% human AB serum at 1000g for 25 minutes at 4°C. This resulted in the separation of the PBMC into two distinct fractions, a cell population at the 40% Percoll gradient interface and a cell pellet at the bottom of the tube. Upon recovery and washing, the two populations appeared to be well fractionated/separated on the basis of size of the cells, whereby the interface consisted predominantly of large sized cells (monocytes and macrophages) while the pellet consisted mainly of small uniform sized cells (lymphocytes). The cell population from the interface was then incubated with anti-CD2 and anti-CD19-coated Dynabeads (DYNAL, Inc., Lake Success, NY) followed by depletion using the DYNAL-MPC-1 magnet as per manufacturer's instructions. This technique always resulted in about 80% of the recovered cells post-depletion being positive for the monocyte surface antigen CD14 with less than 2% each of CD3 cells (T lymphocytes) and CD19 cells (B lymphocytes) as determined by FACS analysis. Similarly the cell population from the

pellet fraction were incubated with a cocktail of anti-CD8, anti-CD14 and anti-CD19-coated Dynabeads and subjected to the DYNAL magnet depletion technique. The resulting cell population recovered from the pellet fraction post-depletion was always greater than 90% positive for the T lymphocyte antigen CD4 as determined by FACS analysis.

Dendritic cell cultures

The highly enriched CD14 monocyte cell population from each patient was cultured in the presence of the cytokines GM-CSF and IL4 (Genzyme Corporation, MA.) at a concentration of 10000U/ml for each cytokine. The cells were cultured in 6-well plates at $2\text{-}3x10^6$ cells per well in 3 ml of CM. These were incubated at 37°C and 5% CO_2 for a period of 7-8 days. FACS analysis of the cells harvested at the end of the culture period indicated them to be enriched for cells displaying antigens consistent with a dendritic cell phenotype. These cells were highly positive for the expression of HLA DR, CD80, CD86, with low expression of CD83 and negative for the expression of CD14 and CD19 antigens.

Proliferation assay

Recall antigen proliferation assays were performed using irradiated DC (3000 cGy, ^{137}Cs source) at $1x10^4$ cells/well in the absence and presence of $1x10^5$ CD4 T cells/well in 96-well U bottom plates (Falcon). Purified preparations of the prostatic proteins PAP, PSA and β-microseminoprotein PSP94 (Fitzgerald Industries, Inc.) was added to the respective wells at 10μg/ml and tetanus toxoid was used at 1:100 dilution of the commercially available product (Connaught Laboratories, Ontario, Canada). Some wells did not have any antigens added to them in order to measure the non-specific or background stimulation of the CD4 T cells by the DC and components of the culture medium. All stimulations were performed in duplicate or triplicate and the plate was cultured for a period of 5 days at 37°C and 5% CO_2. ^3H-thymidine was added at 1

μCi/well on the fourth day of culture and the plates were harvested on day 5 using a Tomtec cell harvester (Wallac Inc., Gaithersburg, MD.) and the counts per minute determined by liquid scintillation counting with the Betaplate System (Wallac Inc.). The stimulation index (SI) was calculated as the mean CPM obtained in the presence of antigen divided by the mean CPM obtained in the absence of antigen for all samples.

Statistical analysis

The stimulation index was calculated for each antigen in prostatitis patients and normal volunteers. A stimulation index greater then 2, meaning that the proliferative response to the three prostatic antigens was greater than 2 times the CPM response in the absence of antigen, was interpreted as a significant response for that prostatic antigen. The number of individuals with a significant SI response to each prostatic antigen was compared between the normal and prostatitis groups using Fisher's exact test.

For the response to the control antigen tetanus toxoid we expected that most individuals would be responsive to this antigen but the degree of response would be variable depending upon time since last vaccination, MHC haplotype and other variables. Since patients and volunteers were expected to be responsive to TT we compared the mean proliferative response of the normal and prostatitis groups using the Wilcoxin Rank Sum test. A P value less than 0.05 was interpreted as excluding the null hypothesis for both tests.

Results

The characteristics of the patients and volunteers are shown in table 1. The median age of the patients was 41 years (range 24 to 57) and the median age of the normal volunteers was 34 years (range 22 to 65).

The results of the recall proliferation assays using prostatic antigens are shown in figure 1. In 5 of the 14 prostatitis patients a proliferation index greater than 2 was observed when PSA was used as the antigen. No response was observed among prostatitis patients to PAP or β-MSP. The stimulation index for normal volunteers did not exceed 2 for any of the three prostatic protein antigens tested. The difference between the response to PSA of prostatitis patients was significantly different from that of normal volunteers ($P = 0.03$, Fisher's exact test). There was no difference between the stimulation indices for PAP or β-MSP when comparing normals to prostatitis patients.

CD4 lymphocytes from normal volunteers and prostatitis patients were responsive to the recall antigen tetanus toxoid (TT) as shown in figure 2. The mean (SEM) proliferation index was 7.0 (4.0) for normals and was 13.5 (10.3) for the prostatitis patients. Hence, CD4 T lymphocytes from both groups had comparable positive proliferative responses to TT.

Discussion

We have previously demonstrated that men with chronic prostatitis/chronic pelvic pain syndrome have evidence of CD4 T cell reactivity with seminal plasma and that the antigen being recognized is derived from the prostate (4). To determine if any of the principal secretory products of the prostate that are secreted into the seminal plasma might be recognized by T cells we examined CD4 T lymphocytes from men with chronic prostatitis/chronic pelvic pain syndrome for a proliferative response to purified prostatic proteins obtained from the seminal plasma. We found that CD4 T cells from some men with chronic prostatitis/chronic pelvic pain syndrome manifested a proliferative response to PSA that was not present in normal male volunteers. This suggests that PSA is being recognized by the immune system in some men with chronic prostatitis/chronic pelvic pain syndrome providing evidence that this disorder represents an autoimmune disease in some patients.

The cause of chronic prostatitis/chronic pelvic pain syndrome is unknown. Infection has long been viewed as the etiology for this problem. Certainly patients with bacterial infection of the prostate do exist. Patients with bacterial infections of the prostate typically respond promptly to therapy with antimicrobial agents. However, most patients with chronic symptoms fail to achieve a durable and lasting remission of symptoms with antimicrobial therapy. In addition, a large body of literature to date has failed to provide convincing evidence that some fastidious organism is responsible for symptoms in a significant proportion of patients. Alternate explanations for chronic prostatitis deserve further study.

The hypothesis that chronic prostatitis/chronic pelvic pain syndrome represents an autoimmune disease in some patients is supported by several observations. First, the chronic, relapsing and episodic nature of symptoms is consistent with an autoimmune etiology. Second, the

prostate is commonly found to contain inflammatory infiltrates when prostate tissue is removed for any reason, typically for prostatic cancer or benign prostatic hypertrophy. (6) The reasons for this inflammation and the implications of its presence are completely unknown. Third, we presented evidence that CD4 T lymphocytes from some men with chronic prostatitis/chronic pelvic pain syndrome manifest a recall proliferative response to seminal plasma (4). Lastly, we have also shown that the pro-inflammatory cytokines TNFα and IL-1β are elevated in the semen of some men with chronic prostatitis/chronic pelvic pain syndrome but not asymptomatic normal men (7). These proximal, pro-inflammatory cytokines are also elevated in the joint fluid in rheumatoid arthritis patients and the inhibition of these cytokines in patients with rheumatoid arthritis has clearly improved symptoms (8). These data are consistent with but do not prove that prostatitis is an autoimmune or autoinflammatory condition in some men.

The hallmark of autoimmunity is the demonstration of an immune response against a normal self antigen. The study of antigens in the prostate that could be the target of a T lymphocyte response has principally occurred as a result of investigations in prostate cancer immunotherapy. The central question of cancer immunotherapy is whether antigens exist in human cancers that can be recognized by the immune system and whether this recognition can be therapeutic in patients with cancer. The description of many such tumor antigens in the past few years, principally in melanoma, led to the surprising finding that many melanoma antigens were derived from normal proteins of melanocyte lineage cells (reviewed in (9)). This suggests that cancer recognition by the immune system is self recognition and that many of the features of a successful cancer immunotherapy will resemble autoimmunity. Hence, the search for immunotherapies for prostatic cancer has included a search for normal self antigens in the prostate. The goal of prostate cancer immunotherapy has therefore included the goal of inducing

autoimmune prostatitis. Thus, our observation that this phenomenon may be occurring in some men with chronic prostatitis is interesting both in the hopes of explaining a baffling chronic condition as well as providing potential targets for prostate cancer immunotherapy.

Several investigators have provided evidence that prostatic antigens can be recognized by T cells. Liu et al (10) demonstrated in rats that vaccination with a syngeneic prostate homogenate could induce a T cell immune response to prostate steroid-binding protein (PSBP). This protein, like PSA in humans, is a major secretory product of the rat prostate but no homolog in humans exists. Vaccination of rats with purified PBSP induced a vigorous antibody and T cell response and induced inflammatory infiltrates destructive to the prostatic epithelium in some animals. Fong et al (11) immunized rats with a vaccinia virus expressing human PAP and demonstrated that an inflammatory prostatitis in the animals could be engendered. A vaccinia construct containing rat PAP could not induce prostatitis in vaccinated animals. This suggests that a T cell response to self prostatic antigens can be induced in rats.

In human studies the culture of T cells demonstrating clear specificity for prostatic antigens has been very difficult. All human tumor antigens recognized by T cells have been identified by the production of such specific T cell lines either directly from human tumors or peripheral blood of cancer patients and the subsequent identification of the antigen being recognized using a variety of techniques. Since prostate specific T cell lines have not been reported by these methods investigators have identified known proteins that are specifically expressed by the prostate and attempted to prove that these proteins or peptides derived therefrom are potential antigens for T lymphocytes. It is possible to induce specific T cell lines from normal volunteers by in vitro stimulation of lymphocytes with synthetic peptides and interleukin-2 to expand reactive T cells recognizing the peptide (12). Using such an approach PSA has been

identified as a potential antigen for T cells, principally in normal volunteers of defined HAL haplotypes (13-15). We examined a group of HLA-A2 prostate cancer patients and found similar reactivity with one PSA peptide in only one of seven patients (16).

Other prostatic protein antigens have been explored as potential targets for T cells. Peshwa et al described CD8 T cell recognition of peptides derived from PAP (17). Prostate specific membrane antigen (PSMA), a transmembrane prostate-specific protein also has been demonstrated to contain antigenic sequences that can be used to generate peptide-specific T cells (18). These preclinical studies have been performed to support various trials of prostatic cancer immunotherapy with antigen pulsed dendritic cells (19; 20) or vaccination with a recombinant vaccinia virus expressing human PSA (21). These early clinical trials are designed to induce a prostate specific immune response presumably directed against metastatic prostate cancer deposits as well as the intact prostate gland. Our data support the notion that PSA may be a target of a human immune response in some men with chronic prostatitis/chronic pelvic pain syndrome suggesting that attempts to target this antigen in prostate cancer may be successful.

In summary, the data are consistent with the interpretation that some men with chronic prostatitis/chronic pelvic pain syndrome have an autoimmune component to their disease and that PSA may be a normal self antigen against which this immune response may be directed. To most clearly show this definitively will require the identification of T cell lines specific for PSA, identification of the PSA peptide epitopes being recognized, the HLA haplotypes used to present the peptides and the determination of precursor frequency of peptide specific T cells in normals and patients through the course of the disease.

Acknowledgement

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Figure legends

Figure 1: Purified CD4 T lymphocytes from normal volunteers (left panel) or prostatitis patients (right panel) were stimulated with irradiated autologous dendritic cells pulsed with purified prostatic antigens as shown. Proliferation of the responder CD4 T cells was determined by the uptake of ^{3}HTdR following a five day co-culture. Proliferation index is the ratio of the counts per minute (CPM) in the presence of antigen divided by he CPM in the absence of antigen. Abbreviations: PAP, prostatic acid phosphatase; PSA, prostate specific antigen; bMSP, beta-microseminoprotein.

Figure 2: Purified CD4 T lymphocytes from normal volunteers or prostatitis were stimulated with irradiated autologous dendritic cells pulsed tetanus toxoid. Proliferation of the responder CD4 T cells was determined by the uptake of ^{3}HTdR following a five day co-culture. Proliferation index is the ratio of the counts per minute (CPM) in the presence of antigen divided by he CPM in the absence of antigen

Tables

Table 1. Demographics of prostatitis patients and normal volunteers

Subject number	Age	Race
Prostatitis patients		
Pr85	56	White
Pr86	57	White
Pr87	32	Hispanic
Pr88	54	White
Pr90	49	White
Pr92	24	Black
Pr94	34	White
Pr98	25	White
Pr101	42	White
Pr102	33	White
Pr103	43	White
Pr104	57	White
Pr105	41	White
Pr106	36	White
Normal volunteers		
ND12	39	Asian
ND13	40	Black
ND14	28	White
ND15	38	White
ND16	65	White
ND17	24	Asian
ND18	22	White
ND20	23	White
ND21	26	White
ND22	31	White
ND23	58	White
ND24	42	White

Figures

Figure 1: Recall proliferation of CD4 T lymphocytes stimulated with autologous DC and purified prostatic antigens.

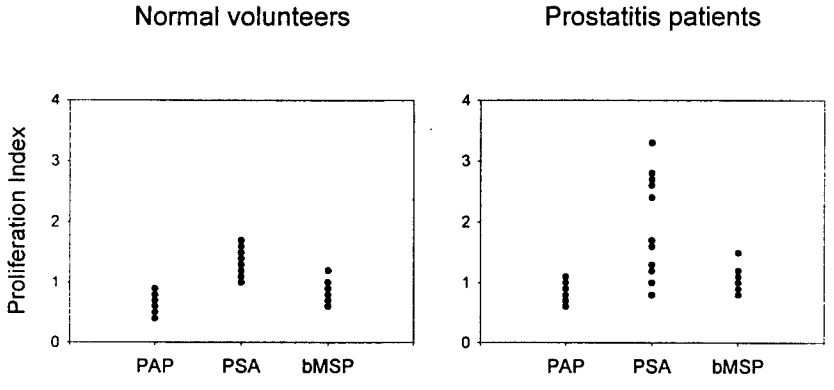
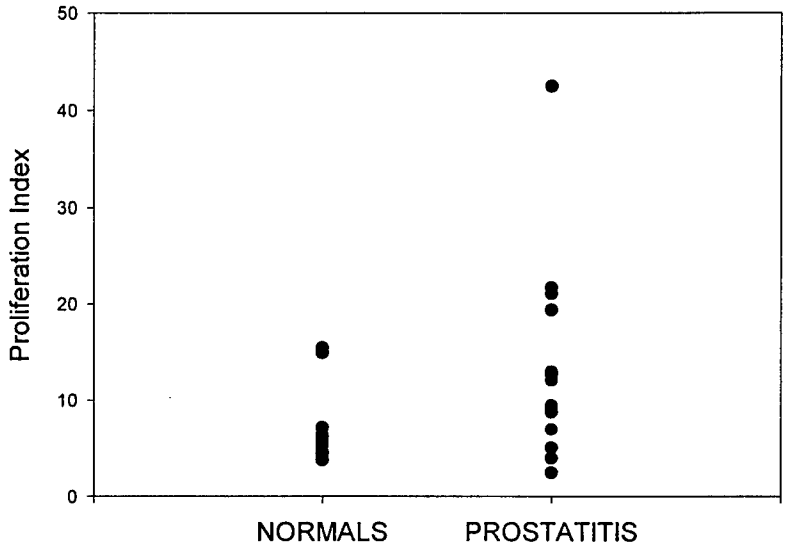


Figure 2. Recall response of CD4 T lymphocytes to tetanus toxoid presented by autologous DC.



Antigen Processing and Presentation Gene Expression in Normal, Primary and Metastatic Prostate Tumor Cell Lines

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Running title: TAP and LMP gene expression in prostate cancer cell lines

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ABSTRACT

In order for immunotherapy to be effective, cytotoxic T lymphocytes must recognize target peptides presented on the tumor cells in the context of major histocompatability complex (MHC) Class I. Defective or diminished expression of antigen processing (LMP-2 and LMP-7) and transporters associated with antigen processing (TAP-1 and TAP-2) genes can result in decreased surface expression of MHC Class I. We utilized reverse transcriptase-polymerase chain reaction (RT-PCR) to analyze LMP-2, LMP-7, TAP-1 and TAP-2 gene expression in normal prostate epithelial and primary prostatic tumor cell lines recently established from the same individual and the longterm cultured metastatic prostate tumor cell lines (DU-145, LNCaP and PC-3). We further investigated the capacity of interferon-γ (IFN-γ) to upregulate the expression of these genes the cell lines. LMP and TAP gene expression was present in cell lines derived from normal prostate epithelial cells and primary prostate tumor cells and upregulated by IFN-γ treatment. Two of the metastatic prostate tumor cell lines (DU-145 and PC-3) expressed LMP and TAP genes and responded to interferon treatment. The third metastatic prostate tumor cell line, LNCaP, had low to undetectable levels of LMP and TAP genes and interferon treatment did not increase the expression of these genes. Our data indicates that prostate tumor cells have intact antigen processing and presenting machinery and should be capable of presenting tumor antigens to cytotoxic T lymphocytes.

INTRODUCTION

The ability of antigen presenting cells (APC) to generate antitumor activity by helper and cytotoxic T lymphocytes depends on their capacity to take up and process potential tumor antigens to peptides. These peptides are presented in the context of major histocompatibility complex (MHC) Class I and Class II molecules and interact with cognate T cell receptor on CD4 and CD8 T lymphocytes. T lymphocytes are capable of recognizing and attacking the tumor cell provided the targeted peptide is expressed by the relevant MHC Class I or Class II molecule on the surface of the tumor cell.

Endogenously synthesized proteins that are potential tumor antigens are processed into peptides by the multicatalytic proteasome complex containing the interferon inducible subunits, LMP-2 and LMP-7 (1). Once generated, the peptides are shuttled across the endoplasmic reticulum by a heterodimeric complex composed of the transporters associated with antigen processing (TAP-1 and TAP-2). Within the ER, the TAP complex is physically associated with nascent MHC Class I molecules and mediates the loading of peptides into the binding cleft of MHC Class I. Downregulated expression of either the proteasome, LMP-2 and LMP-7, or transporter associated with antigen processing, TAP-1 and TAP-2, can result in defective or diminished MHC Class I expression on the surface of tumor cells.

Diminished or absent expression of MHC Class I molecules on the surface of tumor cells is one mechanism whereby a tumor cell can evade the immune system. There are a number of reports where this has been

documented in various tumor cells or in tumor cell lines, including prostate tumor cells (2-4). Sanda et al. reported a loss of MHC Class I expression in two of five metastastic prostate tumor cell lines and under-expression of TAP-2 mRNA in one of the cell lines, PPC (5). Bander et al. utilized immunohistochemistry to analyze MHC Class I cell surface expression with frozen tissue sections of benign prostatic hyperplasia (BPH) and metastatic prostate carcinoma (PCa)(6). Each of the BPH samples was positive for MHC Class I expression, whereas 50% of the PCa were negative for MHC Class I. Modulation of MHC Class I and Class II cell surface expression on metastatic prostate tumor cell lines by interferon-alpha (IFNα) and IFNγ was also determined. PC-3 and DU-145 had baseline levels of MHC Class I and expression was upregulated by IFN treatment whereas, LNCaP expressed low levels of MHC Class I and was not responsive to IFNα or IFNγ treatment. Blades et al. reported a similar finding of normal MHC Class I expression on prostate tissue samples obtained from BPH and allele-specific loss (85%) or absent (34%) MHC Class I expression in metastatic prostate carcinoma tissue.(7)

We extended the research of Sanda et al. by analyzing proteasome (LMP-2 and LMP-7) and transporter associated with antigen processing (TAP-1 and TAP-2) gene expression in three metastatic prostate tumor cell lines (DU-145, LNCaP and PC-3)(5). Normal prostate epithelial, seminal vesicle epithelial and primary prostate carcinoma cell lines derived from two patients undergoing radical prostatectomy (8) were similarly analyzed. The

effect of IFN-γ on antigen processing gene expression in these cell lines was also determined.

MATERIALS AND METHODS

Cell Lines

Immortalized cell lines derived from normal prostate (1532NPTX and 1542NPTX), normal seminal vesicle (1542SVTX) and localized tumor (1532CP2TX and 1542CP3TX) were cultured as previously described (8). These cell lines were created by preparing single cell suspensions from prostate tissue following prostatectomy. The cells were transformed with a recombinant retrovirus containing the E6 and E7 transforming proteins of human papilloma virus serotype 16 and the selectable marker, neomycin phosphotransferase. Clones of the transformed cells were prepared by culturing the cells in conditions of less than one cell/well. Loss of heterozygosity in combination with histochemical and flow cytometric analysis was used to distinguish cell lines derived from tumor cells versus normal prostate epithelium. Metastatic prostate cell lines, LNCaP, PC-3 and DU145 were grown continuous culture in cRPMI (RPMI-1640 (Gibco-BRL, Gaithersburg, MD) supplemented with 10% human AB serum (Gemini Bio-Products, Calabasas, CA)) in T-75 tissue culture flasks.

Induction of Antigen Processing Genes

To determine if IFN-γ upregulated expression of MHC antigen processing and transport genes the cell lines were cultured with recombinant

human IFN-γ (Genzyme, Cambridge, MA). Briefly, immortalized prostate cell lines and metastatic tumor cells lines were cultured in cRPMI with IFN-γ at concentrations of 0, 100 or 500 U/ml for 72 hours. The cells were harvested with trypsin and counted. Two million cells were pelleted by centrifugation. The supernatant was removed and the cell pellets were snap-frozen and stored at -80°C.

RNA Extraction and cDNA synthesis

Total RNA was extracted from the frozen cell pellets per manufacturers instructions (Qiagen, Santa Clarita, CA). Briefly, frozen cell pellets were rapidly thawed in a lysis buffer containing guanidinium isothiocyanate. Genomic DNA was sheared and viscosity was reduced by applying the sample to a shredding column (Qiagen, Santa Clarita, CA). Ethanol was added to the eluate and the sample was applied to a silica-gel-based membrane. The RNA was washed in the appropriate buffers and eluted in sterile H_2O.

RNA was transcribed into cDNA by mixing 3 μl RNA with a cocktail containing: 2.5 U/μl MuLV (Murine Leukemia Virus) reverse transcriptase, 1 U/μl RNAse inhibitor (originally isolated from human placenta), 1 mM each dATP, dGTP, dCTP, and dTTP (deoxyribonucleoside triphosphates), PCR buffer, 5 mM $MgCl_2$, and 2.5 μM random hexamers (Perkin Elmer, Foster City, CA). RNA was reverse transcribed by incubation at 42°C for 15 minutes, denatured by incubation at 99°C for 5 minutes, and cooled to 5°C.

Polymerase Chain Reaction

cDNA was serially diluted (1:10, 1:50, and 1:100) and aliquotted onto 96 well PCR trays in a volume of 2 ul/well. A master mix of 0.2 mM dNTP, PCR buffer, 1.5 mM Mg_2Cl, sterile H_2O, 50 pmol of each primer (Operon Technologies, Alameda, CA) and 1U Taq Gold polymerase (Perkin Elmer, Foster City, CA) were added to each well. Thirty cyles of PCR was performed with the following conditions: 94°C denaturing, 30 seconds; 58°C annealing, 30 seconds; 72°C extension, one minute; at the end of thirty cycles, an additional ten minute extension. The primer sequences for each gene analyzed were as follows: Tubulin (5'TCCTTCAACACCTTCTTCAG, 5'TGGCCTCATTGTCTACCATG); TAP-1 (5'CCGCCTCACTGACTGGA TTC, 5'GCACGTGGCCCATGGTGTTGTTATAG); TAP-2 (5'GCCGAGCA TGAAGTCTG, 5'CCACGCTCTCCTGGTAGATC); LMP-2 (5'GGCAGTG GAGTTTGACGG, 5'GGCTGTCGAGTCAGCATTC); LMP-7 (5'TCTACT ACGTGGATGAACATGG, 5'TTGATTGGCTTCCCGGTACTG); β_2-Microglobulin (5'CCAGCAGAGAATGGAAAGTC, 5'GATGCTGCTTACA TGTCTCG) PCR products were analyzed by 6% polyacrylamide gel electrophoresis stained with ethidium bromide and visualized under an ultraviolet light.

Results

Antigen Processing and Transporter Gene Expression in Normal Prostate Epithelial and Primary Prostate Tumor Cell Lines

Normal prostate epithelial cell lines were assessed for LMP and TAP gene expression by RT-PCR. As shown in figure 1, normal prostate cell lines derived from both patient samples express LMP and TAP genes. Primary

prostate tumor cell lines were derived from the same patients and were similarly analyzed for antigen processing and presentation gene expression. LMP-2, LMP-7, TAP-1 and TAP-2 gene expression was detected in both primary prostate tumor cell lines as shown in figure 2.

Effects of IFN-γ Treatment on Antigen Processing and Transporter Gene Expression in Primary Prostate Tumor and Normal Prostate Epithelial Cell Lines

Expression of LMP and TAP genes can be induced by treatment with IFN-γ. We determined whether IFN-γ would increase the expression of antigen processing and transport genes in normal prostate epithelial and primary prostate tumor cell lines by culturing the cells for forty-eight hours with 100 or 500 U/ml of IFN-γ. Cell lines derived from normal and primary prostate cancer cells were responsive to treatment with IFN-γ as shown in figures 1 and 2, respectively.

Antigen Processing and Transporter Gene Expression in Metastatic Prostate Tumor Cell Lines

In addition to detecting LMP and TAP gene expression in normal and primary prostate tumor cell lines which have been in continuous culture for more than one year, we analyzed LMP and TAP gene expression in three metastatic prostate tumor cell lines which were generated more than twenty years ago. DU-145, LNCaP and PC-3 cells all expressed TAP-1 and TAP-2 (figure 3), although less PCR product was detected in the metastatic prostate cell lines compared to the normal and primary tumor lines. Detectable levels of LMP-2 and LMP-7 were seen in DU-145 and PC-3 cells, again at a lower

intensity than seen for normal and primary prostate cell lines. LMP-2 and LMP-7 gene expression was not detectable in LNCaP cells.

Effects of IFN-γ Treatment on Antigen Processing and Transporter Gene Expression in Metastatic Prostate Tumor Cell Lines

The effect of IFN-γ on LMP and TAP gene expression was also assessed in three metastatic prostate cell lines, DU-145, PC-3 and LNCaP (Figure 3). IFN-γ treatment increased antigen processing and transport gene expression in both DU-145 and PC-3 cells. Consistent with other reports, LNCaP cells were not responsive to IFN-γ.

Discussion

Prostate cancer is the second most common type of cancer found in American men. The American Cancer Society estimates that there will be nearly 200,000 new cases of prostate cancer diagnosed in the United States this year, and about 40,000 men will die of this disease. Primary prostate carcinoma will account for approximately 60% or 120,000 of these newly diagnosed cases. Immunotherapy approaches for the treatment of prostate cancer include generating APC *ex vivo*, pulsing them with known prostate antigens (such as peptides derived from PSMA) and infusing them back into the patient(9, 10). The effectiveness of these treatment modalities is dependent upon the ability of cytotoxic T lymphocytes to recognize tumor peptide antigens presented in the context of MHC Class I molecules on the tumor cells.

One mechanism whereby tumor cells can evade the immune system is by diminished or absent expression of MHC Class I molecules on the surface of tumor cells. Blades et al. assessed surface expression of MHC Class I molecules in prostate samples by immunohistochemistry. A complete loss of MHC Class I expression was seen in 34% of prostate carcinoma samples. No correlation between loss of MHC Class I expression and stage of disease was seen in the study by Blades(7). In contrast, Klein et al. reported intact surface expression of β-2M on 88% of well-differentiated prostate adenocarcinoma whereas only 16% of poorly differentiated tumors were positive(3). We analyzed proteasome (LMP-2 and LMP-7) and transporter associated with antigen processing (TAP-1 and TAP-2) gene expression in normal prostate epithelial, seminal vesicle epithelial and primary prostate carcinoma cell lines derived from two patients undergoing radical prostatectomy (8) by reverse transcriptase-polymerase chain reaction (RT-PCR). We report here that antigen processing gene expression is intact in the cell lines derived from primary prostate tumor carcinoma and normal prostate epithelial cell lines. In addition, these cell lines were responsive to treatment with IFN-γ. In order to determine whether immortalization affected the expression of antigen processing and presentation genes, short-term cultures of normal and primary prostate carcinoma cells were analyzed. Levels of TAP and LMP gene expression were similar to those seen in immortalized cells lines (data not shown).

In addition to the normal and primary prostate tumor cells, we analyzed proteasome (LMP-2 and LMP-7) and transporter associated with antigen processing (TAP-1 and TAP-2) gene expression in three metastatic prostate tumor cell lines (DU-145, LNCaP and PC-3). Our findings extend those of Sanda et al. who reported that TAP-2 gene expression was intact in DU-145 and PC-3 and defective in LNCaP. We show that DU-145 and PC-3 expressed low, but detectable levels of antigen processing genes and were responsive to IFN-γ, whereas LNCaP expressed very low levels of antigen processing gene RNA which was modestly increased by treatment with IFN-γ.

Immunotherapy relies upon CTL recognition of tumor peptide antigens presented by MHC Class I on the surface of tumor cells. Our data show that prostate tumor cell lines should be capable of presenting tumor antigens on MHC Class I and should be targeted by CTL, since primary prostate cancer cell lines and two of three metastatic prostate cancer cell lines analyzed expressed antigen processing machinery and were responsive to treatment with IFN-γ. These data lend support for continued immunotherapy approaches using dendritic cells armed with putative prostate tumor antigens, particularly for the treatment of minimal residual disease following prostatectomy.

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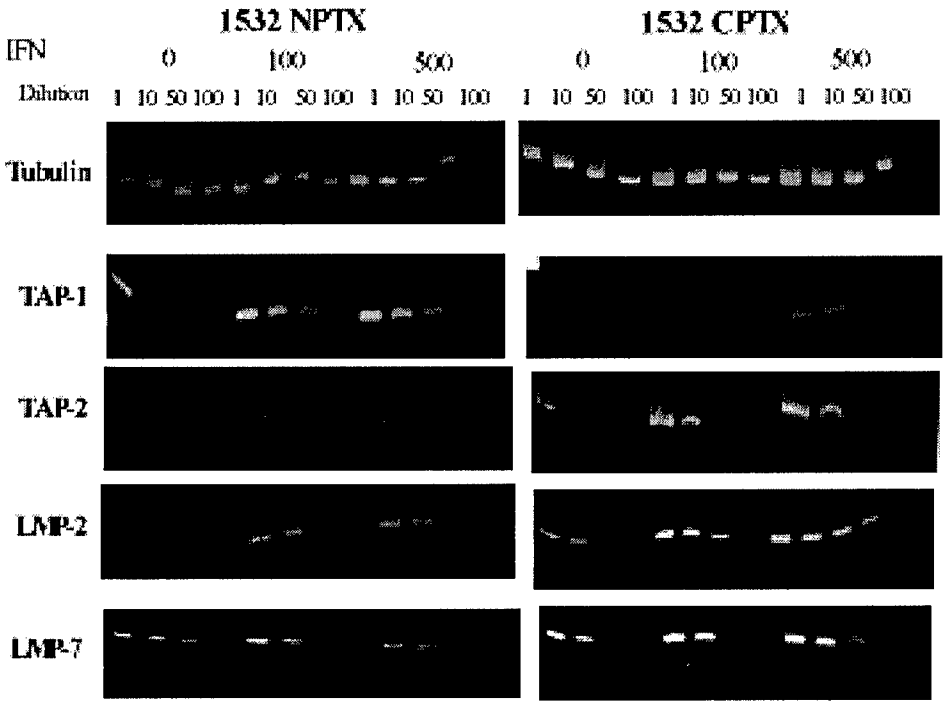
Figure Legends

Figure 1. **TAP and LMP gene expression in normal prostate epithelial cell lines.** Normal prostate epithelial cell lines were established from two patients undergoing radical prostatectomy for primary prostate adenocarcinoma as described in the methods. The cells were plated in six well plates and cultured for 48 hrs. with 0, 100, or 500 U/ml IFN-γ. The cells were harvested and counted. Two million cells were removed and a cell pellet was prepared by centrifugation. The supernatant was removed and the cell pellet was snap frozen and stored <-80°C. Total RNA was isolated from the cell pellets and cDNA synthesized. The cDNA was serially diluted 1:1, 1:10, 1:50, and 1:100 and PCR performed using the primers as indicated. PCR products were analyzed by 6% PAGE, stained with ethidium bromide, visualized under a UV light and photographed. Both patient cell lines expressed LMP and TAP genes shown in lanes 1-4. The cell lines were responsive to treatment with IFN-γ as shown in lanes 5-12. Tubulin, a constitutively expressed gene, was used as a control.

Figure 2. **TAP and LMP gene expression in primary prostate cancer cell lines.** Primary prostate cell lines were established as described in the methods. The cells were plated and cultured with 0, 100 or 500 U/ml IFN-γ. The cells were harvested and analysis of antigen processing and presentation genes was performed as described in figure 1. Lanes 1-4 show that TAP and LMP gene expression was intact in both patient primary tumor cell lines and these lines upregulated gene expression in response to IFN-γ treatment (lanes 5-12).

Figure 3. **TAP and LMP gene expression in primary prostate cancer cell lines.** Longterm metastatic prostate cancer cell lines were cultured with 0, 100 or 500 U/ml IFN-γ and harvested as described in figure 1. DU-145 and PC-3 cells express low but detectable levels of all genes analyzed (lanes 1-4) and were responsive to treatment with IFN-γ (lanes 5-12). LNCaP cells did not express detectable levels of LMP-2 and LMP-7 and was not responsive to treatment with IFN-γ (lanes 5-12).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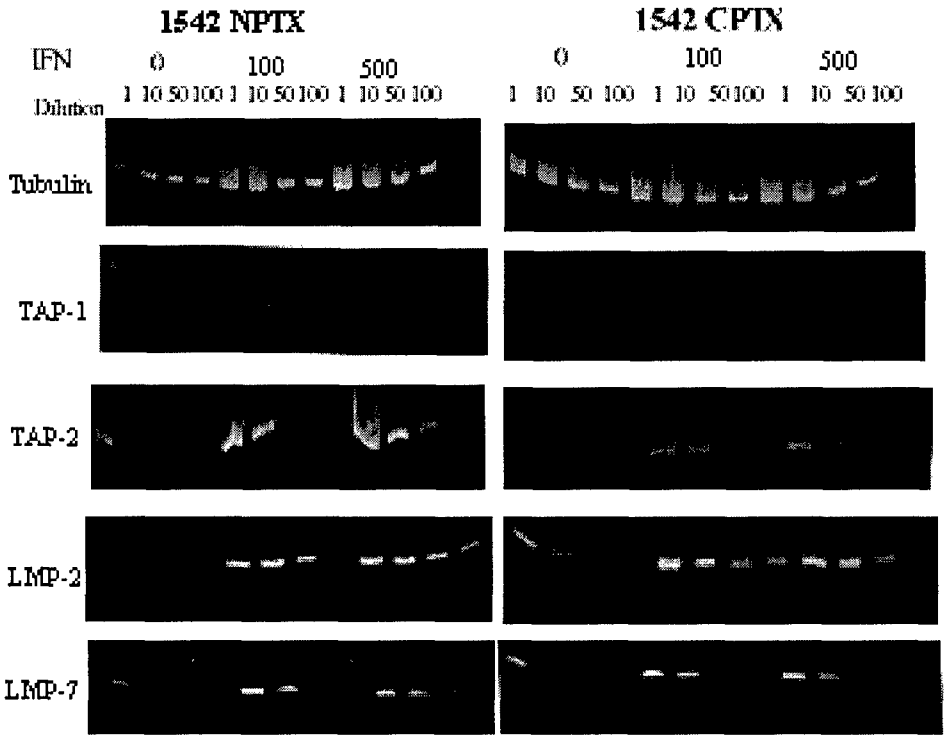
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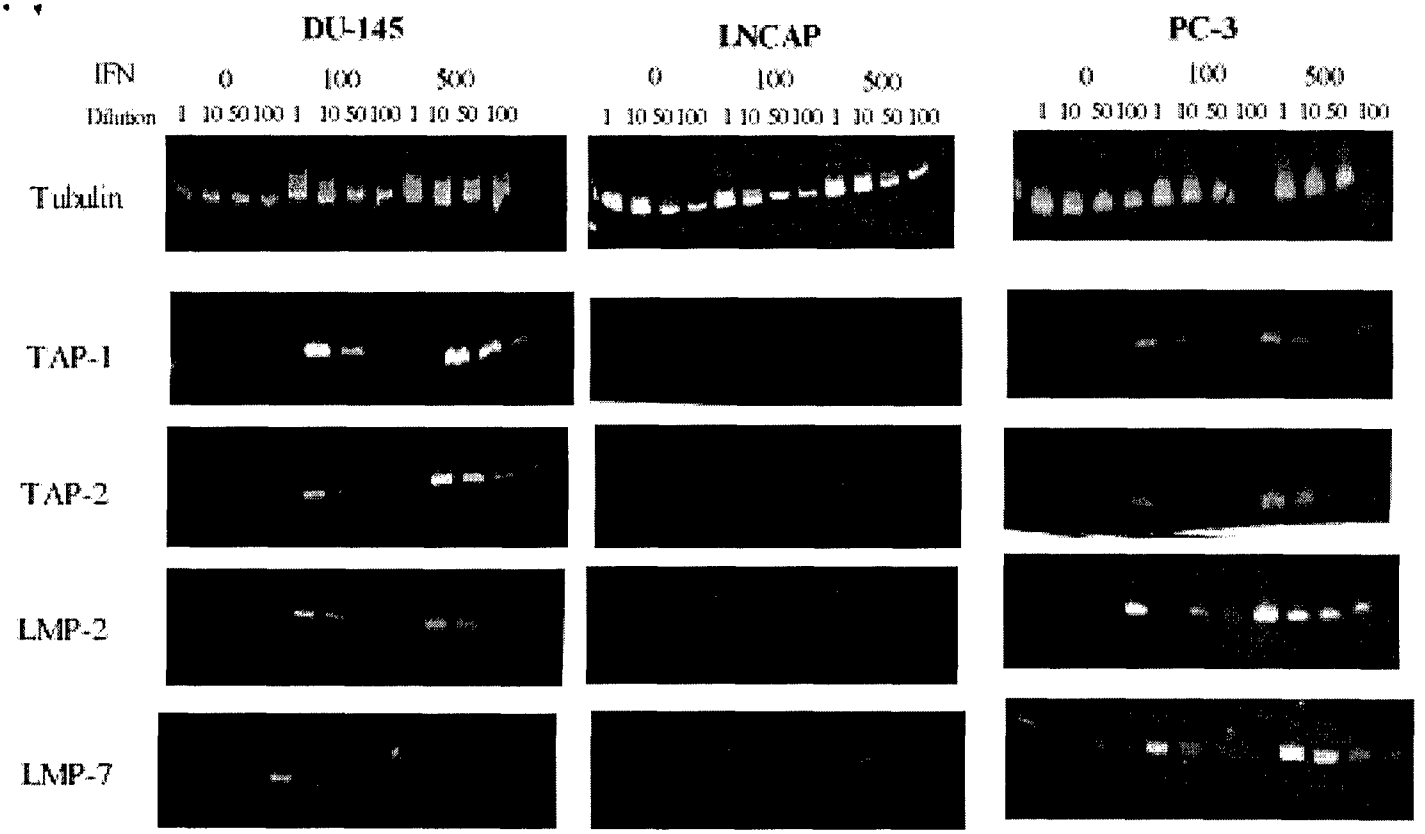
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